REMARKS

Claims 1-38, 42, 43, 58, 59, 73, 79, 82, 89-91, 99 and 113-203 were previously pending in this application. By this amendment, Applicant is canceling claims 141-157, 171-175 and 178-183 without prejudice or disclaimer. Claims 1, 17, 24, 25, 27-31, 38, 42, 43, 58, 59, 73, 79, 91, 113-122, 124, 125, 130-134, 158-161 and 196 have been amended. New claims 204-222 have been added. Support for the newly added claims can be found in the specification on pages 2-8 and 13 as well as in the claims as originally filed. As a result, claims 1-38, 42, 43, 58, 59, 73, 79, 82, 89-91, 99, 113-140, 158-170, 176, 177 and 184-222 are pending for examination with claims 1, 38, 42, 43, 59, 73, 79, 82, 89, 90, 91, 99 and 204 being independent claims. No new matter has been added.

Objections to the Specification

The Examiner has indicated that the essential material in the specification that is incorporated by reference to a foreign application or patent, or to a publication is improper. The Examiner argues that the Applicant is relying on various references to teach essential material and that amendment to the disclosure to include such information is required.

Applicant respectfully disagrees that amendment to the specification is required. Applicant maintains that the publications cited by the Examiner, namely Razi et al., Yates et al., Petitou et al. and Vlodavsky et al., are used to illustrate that the terms "biotechnology derived heparin", "chemically modified heparin" and "synthetic heparin", as well as the compounds to which they refer, are well known to those of ordinary skill in the relevant art. The Applicant wishes the Examiner to note that these references were published before the filing date of the instant application. The references serve to demonstrate that the terms are well known to those of ordinary skill in the art as are the methods of making the compounds to which the terms refer. Therefore, the listing of these references in the specification is not for the purpose of incorporating essential material as the Examiner has asserted.

Accordingly, withdrawal of this objection is respectfully requested.

Objections to the Claims

The Examiner has indicated that claims 113-129, 130-134 and 135-140 are substantial duplicates of claims 141-157, 171-175 and 178-183, respectively. The Examiner has stated that should the former claims be found allowable the substantial duplicate claims will be objected to.

Applicant respectfully thanks the Examiner for pointing out that the claims were duplicated in the claim set prior to this Amendment. Applicant has canceled claims 141-157, 171-175 and 178-183 accordingly.

Rejections under 35 U.S.C. §112

The Examiner has rejected claims 6-10, 32, 121, 123, 149, 151, 162, 185 and 196 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Examiner has maintained that the claims contain subject matter that was not described in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) at the time the application was filed had possession of the claimed invention. Specifically, the Examiner argues that the Applicant has not adequately described how to make "biotechnology derived heparin", "chemically modified heparin", "heparin analogue", "AT-III binding saccharides" and "unfractionated heparin preparations" and has relied on various references to incorporate by reference to teach how to make said products.

Applicant respectfully disagrees. The references are cited to demonstrate the knowledge within the art at the time the instant application was filed and are not cited for the purpose of teaching essential information. Applicant maintains that one of ordinary skill in the art would not only understand what is meant by the aforementioned terms but would also be familiar with methods of their making.

For instance, "biotechnology derived heparin" refers to any heparin molecule that is prepared with any biotechnological method known to one of ordinary skill in the art. The references cited serve to demonstrate that at the time of filing of the instant application, one of ordinary skill in the art was familiar with making heparins through biotechnological means, which include, for example, synthesis of the molecules.

"Chemically modified heparins" refer to any heparin molecule that has been modified chemically. Applicant is herewith providing additional references that further demonstrate that those of ordinary skill in the art were familiar with these molecules as well as methods of making them. For instance, examples of chemically modified heparins as well as methods for their production are provided in Belford et al. (Biochemistry (1992), 31, 6498-6503) and Desai et al. (Carbohydrate Research (1993), 241, 249-259). Belford et al. provide chemically modified heparins that are, for example, N-desulfated, N-acetylated, N-acetoacetylated, totally desulfated, N-resulfated, carboxyl reduced, etc. as well as combinations thereof, which can be prepared by methods that were well known in the art (See, for example, page 6499, second paragraph under **Materials and Methods**). Desai et al. likewise provides examples of chemically modified heparins, which are produced from alkaline or sodium carbonate solutions (See, for example, pages 249 and 258).

Additional references provided herewith further demonstrate that "heparin analogues" were also known in the art at the time of filing of the instant specification. For instance, Casu et al. (Carbohydrate Research (1994), 263, 27-184, **abstract**) provides a polysaccharide modified by N-deacetylation, N-

sulfation and O-sulfation, which exhibits heparin-like properties, while Torngren et al. (Thromb. Res. (1990), 59, 237-46, **abstract**) and Beales et al. (Diabetes Res. Clin. Pract., 1993, 21, 5-9, **abstract**) also provide examples of heparin analogues.

Furthermore, examples of saccharides that bind antithrombin III (AT III) were also known in the art at the time of filing of the instant application. This is further evidenced by, for example, Petitou et al. (Bioorg. Med. Chem. Lett.(1999), 9, 1155-60, **abstract**) and Basten et al. (Bioorg. Med. Chem. Lett. (1998), 8, 1201-6, **abstract**), each of which provides examples of heparin mimetics that contain AT III binding domains.

Finally, "unfractionated heparins", which are recognized by those of ordinary skill in the art to be heparins that have not undergone a fractionation procedure, are also well known in the art. Ruiz-Calero et al. (J. Chromatogr. A. (1998), 828, 497-508, **abstract**) and Gonzalez E. R. (Pharmacotherapy (1999), 19, 155S-160S, **abstract**) both demonstrate the use of the term "unfractionated heparins" in the art at or before the filing of the instant application

Therefore, as provided in the specification and further evidenced by the above, at the time of the application filing, it is clear from these references that those of ordinary skill in the art were not only familiar with the terms "biotechnology derived heparin", "chemically modified heparin", "heparin analogue", "AT-III binding saccharides" and "unfractionated heparin preparations" but were also familiar with methods to make them. Applicant respectfully reminds the Examiner that possession merely requires that the claimed invention be described in a way such that the meaning of the terms would have been clear to one of ordinary skill in the art at the time the application was filed. Therefore, as these terms were known to those of ordinary skill in the art at the time of the application filing, as evidenced by the references, and necessarily clear, the written description requirement is satisfied.

Accordingly, withdrawal of this rejection is respectfully requested.

The Examiner has also rejected claims 17-30 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Examiner has argued that these claims limit the subjects to be treated to those that are "at risk" of developing various disorders, but the specification does not disclose any methods of determining if a subject is "at risk of atherosclerosis" or "at risk of an inflammatory disorder". The Examiner further argues that the specification does not provide an adequate written description of the risk factors associated with all of the various disorders.

Applicant respectfully disagrees. It is well within the knowledge of those of ordinary skill in the medical arts to determine not only who has a disorder but also to determine who is at risk for suffering from the disorder. Further, Applicant maintains that based on the disclosure provided in the specification

as well as the knowledge of those in the relevant art, the Applicant at the time of the filing of this application adequately demonstrated possession of the claimed invention. The meaning of the claims would have been sufficiently clear to those of ordinary skill in the art at the time of the application filing.

However, in the interest of expediting the prosecution of this application, Applicant has amended the claims so that the claims encompass treating subjects that have the various disorders of the claims. The Applicant wishes the Examiner to note that prior to this amendment the claims encompassed treating subjects that have the disorders of the claims in addition to those at risk of suffering from the disorders.

Accordingly, withdrawal of this rejection is respectfully requested.

The Examiner has also rejected claims 1-38, 58-59, 73, 82, 89-91, 113-115, 121, 123, 126, 127, 130-143, 149, 151, 154, 155, 158-175, 178-190 and 196-198 under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

In regard to claim 1, the Examiner has rejected the claim as the term "effective amount" is indefinite where the claims fails to state the function that is to be rendered effective. Applicant respectfully disagrees; however, in order to expedite the prosecution of this application, Applicant has amended claim1 to remove the recitation of "effective". The rejection of this claim is, therefore, now moot.

In regard to claim 6, where the claim provides that "wherein the heparin is a biotechnology derived heparin", the Examiner has indicated that the claim is indefinite as it is unclear as to how the recitation of the source of the active agent further limits the method. Applicant again disagrees. As argued previously, claim 6 narrows the scope of claim 3 from which it depends. The method of claim 3 encompasses the administration of heparin regardless of its source; however, claim 6 encompasses only the administration of biotechnology derived heparin. This would clearly exclude the administration of heparin obtained, for example, from a natural source, such as porcine mucosa. Therefore, the claim scope between the claims are different, and the difference would be distinguishable to one of ordinary skill in the art.

The Examiner has also rejected the reference to "a chemically modified heparin", as the claim does not set forth how the heparin is modified and to what extent the heparin is modified. The Examiner concludes that without identifying the moieties intended to modify heparin, the identity of "a modified heparin" would be difficult to ascertain. Applicant respectfully disagrees. Applicant maintains that one of ordinary skill in the art would recognize the ways in which heparin can be chemically modified. In addition, Applicant maintains that one of ordinary skill in the art would recognize such chemically

modified molecules. The term is intended to encompass heparins that are modified chemically in any of a number of ways but not such that the chemical core of heparin is unrecognizable, and as the Examiner has pointed out, heparin is an art recognized compound with an art recognized chemical core. Therefore, chemically modified heparins would be easily ascertained by those of ordinary skill in the art as chemically modified heparins would contain the recognizable chemical core. In addition, as evidenced by at least Yates et al., Belford et al. and Desai et al. examples such molecules were known to those of skill in the art at the time of filing of the instant application. Therefore, Applicant maintains one of ordinary skill in the art would be able to determine the metes and bounds of the claim.

The Examiner has also rejected claim 31 as there is insufficient antecedent basis for the limitation "the angiogenic disorder". Applicant thanks the Examiner for pointing this out and has amended claim 31 so that it depends from claim 30 rather than claim 2.

The Examiner has rejected claim 32 for being indefinite as the identity of the moieties intended to modify the art recognized chemical core of pectin would be difficult to ascertain. Applicant respectfully disagrees. It is common within the chemical arts to produce derivative molecules based on a chemical core. In addition, pectin derivatives contain an art recognized core, and as derivatives would contain such a core, one of ordinary skill in the art would be able to identify the derivative molecules. One of ordinary skill in the art would, therefore, be able to determine the metes and bounds of the claim.

The Examiner has also rejected claims, such as claim 38 and 113-115, for the phrase "delivering at least 5% of a polysaccharide" or some other percentage, which the Examiner maintains is unclear. Applicant respectfully disagrees and maintains that the claims are sufficiently clear. The claims clearly are directed to methods for the administration of polysaccharides via the use of particles or a dry aerosol. Based on the plain language of the claims, it would be clear to one of ordinary skill in the art that the percentage refers to the percentage of the polysaccharides out of the total of the polysaccharides administered, whether in particle or aerosol form. Applicant asserts that the language of the claims would be sufficiently clear to one of ordinary skill in the art.

The Examiner has also rejected claim 58 as being indefinite for administering the composition of claim 43, which is drawn to a glycosaminoglycan rather than a composition. Claim 58 has been amended to correct for this typographical error.

The Examiner has also rejected claims 59 and 73 for use of the word "rapidly". The Examiner maintains that the word is not defined in the claims. Although Applicant again disagrees, as the claim specifically provides the timing to which "rapidly" refers, Applicant has amended the claims to expedite the prosecution of this application.

The Examiner has also rejected claims 82, 89 and 90 for the phrase "heparin-like glycosaminoglycan" (HLGAG), which the Examiner maintains renders the claims indefinite as one of ordinary skill in the art would not be reasonably apprised of the scope of the invention. Applicant again disagrees. The term HLGAG is a well-known term of art that has been in use since as early as 1978 according to a brief review of the references available in the PubMed database. A demonstration that a term is an art-recognized one necessarily negates the assertion that the term is indefinite. In addition, although not necessary, Applicant has defined HLGAG in the specification on page 20, lines 28-30, as a "family of molecules with heparin like structures and properties". This definition is not only consistent with the use of the term in the relevant art, but also clearly provides the molecules that are encompassed by the term (i.e., molecules that have structures and properties like that of heparin, which are well known in the art). Therefore, as the term is well-known in the art and defined in the specification by Applicant, one of ordinary skill in the art would know what is meant by the use of HLGAG in the claims and, therefore, be apprised of the scope of the claimed invention. Furthermore, Applicant wishes to note that the claims must be read in light of the specification. Definitions of terms are not limitations that must be read into the claims but rather merely provide their meaning.

The Examiner has rejected claim 91 for being drawn to a kit that includes a "detection system". The Examiner argues that it is unclear as to what is a "detection system". The Examiner again states that although the term is defined in the specification, such a definition will not substitute for defining the same in the claim. Although Applicant disagrees, claim 91 as been amended. Applicant wishes to point out that "detection system" is meant to encompass any detection system that could be used by one of ordinary skill in the art. To require the recitation of one particular detection system would be unfairly and unnecessarily limiting. Applicant maintains that the plain English of the claim as written is sufficiently clear. Applicant again points out to the Examiner that the claim must be read in light of the specification.

The Examiner also rejected claims 126 and 159 for reciting "a polymer to effect slow release of the glycosaminoglycan". The Examiner argues that the claim fails to point out the identity of the polymer. The Examiner also argues that it is unclear as to what to "effect a slow release" means as it could increase or decrease the slow release of the glycosaminoglycan. Based on the plain English of this phrase it is clear that the polymer is intended to result in the slow release of the glycosaminoglycan. In addition, slow release polymers are well known in the art, and a multitude of examples of such polymers are provided in the specification, such as on page 16. Therefore, Applicant is entitled to the genus of such polymers as recited in the claims.

Finally, the Examiner has rejected claim 196 for reciting broadly that the polysaccharide is a glycosaminoglycan and also reciting a narrow list of glycosaminoglycans to which the polysaccharide is

limited. Although the Applicant maintains that the claim prior to this amendment was sufficiently clear and that the claim encompasses only the list of glycosaminoglycans recited, Applicant has amended the claim to expedite the prosecution of this application.

Based on the arguments amendments described above, the Examiner is respectfully requested to withdraw the rejection of the claims under 35 U.S.C. §112, second paragraph.

Rejections Under 35 U.S.C. §102

The Examiner has rejected claims 1-3, 5, 8, 11, 12, 14-32, 36-38, 42, 43, 58, 59, 73, 79, 89-91, 99, 113-119, 121-123, 125, 126, 130-134, 141-147, 149-151, 153, 154, 158-163, 168-177, 184-198 and 203 under 35 U.S.C. §102(b) as being anticipated by Illum et al. (WO 97/35562). The Examiner argues that Illum et al. discloses microspheres and methods of their use made from polysaccharides with an aerodynamic diameter of between 1-10 microns and further provide an unformulated polysaccharide as well as formulated microspheres.

Applicant respectfully traverses the rejection of the claims. The Applicant wishes to point out clear differences between the microspheres of Illum et al. and the invention of Applicant's claims. It is important to note that Illum et al. does not provide a positive teaching that it would be desirable to administer an unformulated dry polysaccharide to produce a therapeutic effect due to the polysaccharide. The polysaccharides of the compositions provided in Illum et al. are used only as a carrier and not as a therapeutic agent as provided in Applicant's claims. One of Applicant's discoveries was that a polysaccharide in an unformulated dry form could be administered to achieve a desired therapeutic effect. Illum et al. provides polysaccharide microspheres that merely serve as carriers to encapsulate a drug. It is the drug rather than the polysaccharide that has the desired therapeutic effect. These teachings, therefore, do not provide the compositions and methods of Applicant's claims. Illum et al. does not directly provide, nor would one of ordinary skill in the art be guided by the teachings of Illum et al. to make or use, unformulated polysaccharide particles to produce a therapeutic effect as provided by Applicant.

In addition, there is no positive recitation of using any of the specific glycosaminoglycans provided by Applicant in any composition or method as asserted. One of ordinary skill in the art would not single out any specific glycosaminoglycan to make an unformulated dry particle or a dry aerosol formulation based on the teachings of Illum et al. Illum et al. only recites heparin and low molecular weight heparin (LMWH) as two in a long list of drugs that can be encapsulated by the polysaccharide microspheres provided. Heparins in unformulated dry form or in a dry aerosol formulation as provided by Applicant are not provided by Illum et al. There is also no guidance provided that would lead one of ordinary skill in the art to single these molecules out and make or use them as provided by Applicant.

Furthermore, Illum et al. teaches that the microspheres must have a size that is less than 10 microns, and preferably less than 5 microns (See, for example, page 10 of Illum et al.). Applicant has made the important discovery that unformulated dry polysaccharide particles can be of any size for administration to produce a therapeutic effect of the polysaccharide. The teachings of Illum et al. do not provide polysaccharide microspheres of any size other than less than 10 microns.

Accordingly, withdrawal of this rejection is respectfully requested.

Rejections Under 35 U.S.C. §103

The Examiner rejected claims 1-38, 42, 43, 58, 59, 73, 79, 82, 89-91, 99 and 113-203 under 35 U.S.C. §103(a) as being unpatentable over Illum et al. The Examiner maintains that wherein the claims cover microspheres of a diameter greater than 10 microns or made of various heparins, it would have been obvious to use particles of larger size and to make various microspheres of other polysaccharides such as other heparins.

Applicant respectfully disagrees. There is no evidence that one of ordinary skill in the art would be motivated to make microspheres with a diameter greater than 10 microns as asserted. In fact, there is evidence to the contrary. As provided on page 12 of the instant specification, it was generally accepted, before Applicant's invention, that larger sized particles were not desirable for pulmonary administration as they were thought to be excessively deposited in the upper airways. Even Illum et al. seems to follow this thinking as they specifically point out that their particles must be less than 10 microns, and preferably, should be less than 5 microns. Sufficient evidence of why one of ordinary skill in the art would have been motivated to produce such particles is not provided by the Examiner.

Additionally, there is no evidence that one of ordinary skill in the art would have been motivated to substitute the polysaccharides of their teachings with heparins as the Examiner has asserted. It is important to note that Illum et al. provide polysaccharides microspheres that are only meant to serve as a carrier for a drug. In fact, Illum et al. only refers to heparin and low molecular weight heparin in a long list of drugs to be encapsulated by the carrier polysaccharide. There is no positive teaching that would lead one of ordinary skill in the art to single out heparin or low molecular weight heparin to make the particles or use them in the methods as provided by the Applicant. Again, the Examiner has not provided sufficient evidence to demonstrate why one of ordinary skill in the art would be motivated to use heparin as provided by Applicant.

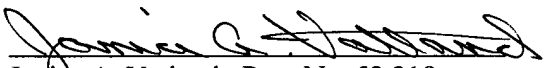
Accordingly, withdrawal of this rejection is respectfully requested.

CONCLUSION

A Notice of Allowance is respectfully requested. The Examiner is requested to call the undersigned at the telephone number listed below if this communication does not place the case in condition for allowance.

If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

Respectfully submitted,
Dongfang Liu et al., Applicant

By: 

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Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Seed, B., & Aruffo, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3365–3369.
Smigel, M. (1986) *J. Biol. Chem. 261*, 1976–1982.
Smith, S. J., & Augustine, G. J. (1988) *Trends Neurosci. 11*, 458–464.
Tang, W. J., Krupinski, J., & Gilman, A. G. (1991) *J. Biol. Chem. 266*, 8595–8603.
Valverde, I., Vandermeers, A., Anjaneyulu, R., & Malaisse, W. J. (1979) *Science 206*, 225–227.
Von Hungen, K., & Roberts, S. (1973) *Nature New Biol. 242*, 58–60.
Walters, E. T., & Byrne, J. H. (1983) *Science 219*, 405–408.
Westcott, K. R., LaPorte, D. C., & STorm, D. R. (1979) *Proc. Nat. Acad. Sci. U.S.A. 76*, 204–228.
Xia, Z., Cheryl, D. R., Merchant, K. M., Dorsa, D. M., & Storm, D. R. (1991) *Neuron 6*, 431–443.
Yeager, R. E., Heideman, W., Rosenberg, G. B., & Storm, D. R. (1985) *Biochemistry 24*, 3776–3783.
Yeager, R. E., Nelson, R., & Storm, D. R. (1986) *J. Neurochem. 47*, 139–144.

Ability of Different Chemically Modified Heparins To Potentiate the Biological Activity of Heparin-Binding Growth Factor 1: Lack of Correlation with Growth Factor Binding

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ABSTRACT: A range of chemically modified heparins was examined for their ability to bind heparin-binding growth factor 1 (HBGF-1; acidic fibroblast growth factor) and potentiate the in vitro mitogenic and neurotrophic activity of HBGF-1. It was found that carboxyl-reduced heparin bound HBGF-1 as effectively as the native heparin molecule. Totally desulfated heparin and N-desulfated heparin lack HBGF-1-binding capacity, and substitution of the exposed amino group with acetyl or acetoacetyl groups only partially restored binding capacity, indicating that *N*-sulfates only play a limited role in growth factor binding. However, the failure of totally desulfated, N-resulfated heparin to interact with HBGF-1 demonstrated that *N*-sulfates alone are insufficient and ester sulfates are absolutely essential for HBGF-1 binding. In contrast, the ability of the modified heparins to potentiate the mitogenic activity of HBGF-1 correlated only to a limited extent with their affinity for HBGF-1. Thus, the carboxyl-reduced molecule which displayed similar affinity for HBGF-1 as native heparin was consistently less potent in augmenting mitogenesis. Similarly, the N-acetylated and the N-acetoacetylated species, which had much lower affinity for HBGF-1 than the carboxyl-reduced molecule, conferred similar biological activity to HBGF-1 whereas N-desulfated heparin, which was unable to bind growth factor, potentiated the mitogenic activity of HBGF-1 for both 3T3 and HUVE cells. In contrast, the neurotrophic activity of HBGF-1 was potentiated by modified heparin species which failed to bind HBGF-1 and were without activity in the mitogenic assays. In fact, native heparin was much less effective at potentiating the neurotrophic activity of HBGF-1 than several of the modified heparins. Thus, heparin exerts its effects not only by binding of HBGF-1 but also by mechanisms independent of its binding activity probably via cell-surface heparin receptors.

Heparin and heparan sulfate proteoglycans (HSPG)[1] are involved in a range of biological functions, including cell–cell (Cole et al., 1986) and cell–substratum (Culp et al., 1980) adhesion, cellular proliferation and differentiation (Fritze et al., 1985), neurite outgrowth (Hantaz-Ambroise et al., 1987), synaptic function (Anderson & Fambrough, 1983), myelination (Carey et al., 1987), matrix assembly (Laurie et al., 1986), in vivo coagulation (Marcum & Rosenberg, 1989), and capillary permeability (Farquhar, 1981).

Heparin will bind to—and in many cases alter the biological activity of—a number of protein and glycoprotein ligands. Heparin is now known to potentiate the mitotic (Schreiber et al., 1985), chemotactic (Terranova et al., 1985), neurotrophic (Unsicker et al., 1987), and angiogenic (Lobb et al., 1985) properties of the pure acidic mitogen heparin-binding growth factor 1 (HBGF-1; also known as acidic fibroblast growth factor or aFGF), and heparin affinity chromatography forms the basis of the purification of HBGF-1 (Maciag et al., 1984).

The binding of heparin to HBGF-1 acts to potentiate its biological activity (Burgess & Maciag, 1989). Indirect evidence for such a mechanism has been derived from experiments that show heparin protects HBGF-1 against proteolytic attack (Lobb, 1988), as well as acid and heat denaturation

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HBGF-1, heparin-binding growth factor 1; HSPG, heparin and heparan sulfate proteoglycan(s); HUVE, human umbilical vein endothelial cell(s).

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(Gospodarowicz & Cheng, 1986), and increases the in vitro biological half-life of HBGF-1 from 7 to 36 h (Damon et al., 1989). The increased in vitro activity of HBGF-1 in the presence of heparin can be reproduced by the more frequent addition of HBGF-1 alone (Rosengart et al., 1988). This, together with data showing the binding of heparin induces a conformational change in HBGF-1 (Schreiber et al., 1985), suggests heparin confers structural stability and/or physically covers the sites of proteolytic attack.

Several studies have more directly linked the potentiating action of heparin to its ability to bind HBGF-1. Gordon et al. (1989) found that 40% of HSPG extracted from the ECM of cultured endothelial cells bound to an HBGF-1 affinity column and this fraction was some 100-fold more active in potentiating the mitogenic action of HBGF-1 than heparin. The flow-through fraction inhibited HBGF-1-induced mitogenesis of endothelial cells. The two fractions were found to differ in size of both their core proteins and their GAG chains. Other studies have shown that decreasing the affinity of HBGF-1 for heparin by proteolytic or chemical modification produces a corresponding decrease in the affinity for the HBGF-1 receptor and a decrease in mitotic activity (Lobb, 1988). Further, Schrieber et al. (1985) have provided compelling evidence that the modulation of HBGF-1 activity by heparin also occurs at the receptor level. Heparin enhanced the binding of HBGF-1 to LEII cells, decreasing the apparent K_d some 2.5-fold. This corresponded to a 5-fold decrease in the EC_{50} for the stimulation of thymidine uptake.

A previous report emphasized the importance of both the polysaccharide chain length and the degree of sulfation on the ability of heparin to potentiate HGBF-1 action (Sudhalter et al., 1989). Highly sulfated oligosaccharides were found to potentiate HBGF-1-induced mitogenesis to a degree proportional to chain length; oligosaccharides (up to tetradecasaccharides) of low sulfate content were inactive. Despite these earlier studies, there has been no systematic analysis of the structural features of heparin required to potentiate HBGF-1 action. In this study, we have attempted to correct this deficiency by examining the ability of a range of chemically modified heparins to (i) bind HBGF-1, (ii) potentiate the mitogenic action of HBGF-1 on both BALB/c 3T3 and HUVE cells, and (iii) potentiate the neurotrophic action of HBGF-1 on dissociated ciliary neurons of the E8 chick.

Materials and Methods

Reagents. Beef heart-derived HBGF-1 was purified as previously described (Watters & Hendry, 1987) except the heparin–agarose column was washed and eluted with a gradient of NH_4HCO_3, allowing immediate lyophilization and storage of the pure factor. Preparations were routinely checked for purity by SDS–PAGE.

Native bovine lung heparin was obtained from Sigma Chemical Co., St Louis, MO. A number of chemically modified heparins were synthesized, namely, (a) N-desulfated, (b) N-desulfated, N-acetylated, (c) N-desulfated, N-acetoacetylated, (d) totally desulfated, (e) totally desulfated, N-acetoacetylated, (f) totally desulfated, N-resulfated, and (g) carboxyl-reduced. Heparin was carboxyl-reduced, N-desulfated, and N-acetylated according to previously published methods (Nagasawa & Inoue, 1980a,b; Irimura et al., 1986; Taylor et al., 1976). Heparin was N-acetoacetylated with acetoacetic anhydride under identical reaction conditions used for acetylation (Irimura et al., 1986). However, totally desulfated heparin was prepared by a modified procedure which resulted in more effective desulfation than previous methods (Nagasawa & Inoue, 1980a,b). Initially, N-desulfated, N-acetoacetylated heparin was converted to its pyridinium salt and totally desulfated in DMSO–10% methanol as reported earlier (Nagasawa & Inoue, 1980a,b). Acetoacetyl groups were removed by the addition of hydroxylamine (50 mg/mL), adjusting the pH of the solution to 7.5 with 5 N NaOH and incubating the mixture at room temperature for 45–60 min. The totally desulfated heparin was exhaustively dialyzed, lyophilized, and, if necessary, N-resulfated under alkaline conditions with pyridine–sulfur trioxide (Irimura et al., 1986).

The sulfate content of the chemically modified heparins was determined by turbidimetry of inorganic sulfate released by acid hydrolysis (Chandrasekaran & BeMiller, 1980), and the content of amino groups was determined fluorometrically using fluorescamine (Weigele et al., 1972) with glucosamine as the standard. The anticoagulant activity of the heparin preparations was estimated by the activated partial thromboplastin time and thrombin time tests as previously described (Parish et al., 1987).

Binding Assay of HBGF-1 to Modified Heparins. The relative affinities of the modified heparins for HBGF-1 were determined using a binding assay based on competition for heparin–agarose (Bio-Rad, Richmond, CA). Each modified heparin was added at various concentrations (up to 500 μg/mL) to a microfuge tube containing 4 μg of HBGF-1 in PBS. Each tube then received 40 μL of a 50% (v/v) suspension of heparin–agarose in PBS to make a final volume of 200 μL. After 1-h agitation at 4 °C, each heparin–agarose pellet was washed 3 times in PBS followed by the addition of 25 μL of SDS–PAGE sample buffer containing 15 mg/mL DTT. Samples were boiled for 3 min, then applied to a 14 cm × 10 cm × 0.75 mm polyacrylamide gel, and electrophoresed at a constant current of 16 mA for 3 h. Gels were stained with Coomassie Blue and HBGF-1 bands quantified by two-dimensional laser densitometry (Ultrascan XL, LKB, Bromma, Sweden). Each band was outlined and its total density integrated using Gel Scan XL version 1.2. For each gel, HBGF-1 bands were normalized to the average of four standards (4 μg of HBGF-1) included on the gel and expressed as a percentage of the total 4 μg added remaining bound to the heparin–agarose beads. Thus, 100% represents 4 μg of HBGF-1 bound to the beads, and 0% is total inhibition of binding. Concentrations giving 50% inhibition of binding were determined from a linear regression formula.

Mitogenic Assays. Stock cultures of BALB/c3T3 cells (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) were maintained in Dulbecco's modified Eagle's medium (DMEM; Flow Labs, McLean, VA)/HCO_3/10% fetal calf serum (FCS; Commonwealth Serum Laboratories) at 37 °C (5% CO_2 incubator) in 60-mL flasks (Corning, NY). Prior to confluency, cultures were lightly trypsinized (0.025%; Worthington Biochemical Corp., Freehold, NJ), resuspended at 15000 cells/mL, and replated. Experimental cultures were seeded into 96-well plates (Nunc, Copenhagen, Denmark) and allowed to reach confluency (4–5 days), at which time the medium was replaced by serum-free DMEM/HCO_3. After 24-h serum deprivation, either HBGF-1 was added at the concentration shown in the presence of a 50 μg/mL sample of the indicated modified heparin or dose responses to the modified heparins were set up in the presence of 10 ng/mL HBGF-1. Cultures were incubated for a further 24 h at 37 °C (5% CO_2) before the addition of [^{3}H]thymidine (Amersham, U.K.). After a further 24-h incubation, the cultures were frozen and thawed twice and harvested onto glass fiber filters (Enzo Biochemicals Inc., NY) using a Titertek 530 cell harvester (Flow Labs), and the radioactivity was counted.

Pooled data from four separate duplicate experiments were analyzed.

HUVE cells (passages 5–12) were isolated and maintained in medium 199 (Flow Labs), 16% fetal calf serum, 25 μg/mL endothelial cell growth supplement (Sigma), and 50 μg/mL heparin. Mitogenic assays were carried out on quiescent cells in the presence of 16% fetal calf serum. Dose responses to the modified heparins were set up in the presence of 10 ng/mL HBGF-1, and [^{3}H]thymidine incorporation was determined as described above.

Neuronal Bioassay. Cultures of E8 chick ciliary neurons were established in collagen-coated 96-well microtiter plates (Nunc) as previously described (Bonyhady et al., 1980, 1982). Briefly, ganglia were dissected aseptically, dissociated with trypsin (0.08%; Worthington Biochemical Corp.) in calcium- and magnesium-free Hank's balanced salt solution, and suspended in DMEM buffered to pH 7.4 with 0.5 M *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES) and containing 1% horse serum (Commonwealth Serum Laboratories). Dissociates were routinely preplated to remove nonneuronal cells. A dose response to the modified heparins was performed in the presence of a constant concentration (25 ng/mL) of beef heart-derived HBGF-1. Heparin samples to be assayed were diluted in the growth medium and added to the top well in a final volume of 100 μL, and a dilution series was set up across the plate. Neuronal cell suspension (100 μL; approximately 2200 neurons) was added to each well, and duplicate cultures were incubated for 24 h at 37 °C in a humidified air incubator. Cytosine arabinoside (10^{-5} M) was incorporated into some cultures to suppress the proliferation of nonneuronal cells. Neuronal survival was assessed by phase-contrast microscopy (200× magnification), the number of phase-bright adherent cells in four fields (approximately 11% of the total area) across the diameter of each well being recorded.

Results

Chemical Modification of Heparins. Seven chemically modified variants of bovine lung heparin were prepared and assessed for their ability to bind HBGF-1 and potentiate the biological activity of the growth factor. The sulfate content, amino group content, and anticoagulant activity of each heparin preparation were assessed and compared with predicted values. It was found that the sulfate content of native heparin was 32.1%; N-desulfated heparin 23.4%; N-desulfated, N-acetylated 22.8%; N-desulfated, N-acetoacetylated 19.8%; totally desulfated <0.2%; totally desulfated, N-acetoacetylated <0.2%; totally desulfated, N-resulfated 12.3%; and carboxyl-reduced 31.5%. These results indicate that removal and addition of sulfate groups were virtually complete. Assessment of the amino group content of the different heparin preparations confirmed this conclusion. N-Desulfation resulted in approximately 80% exposure of the amino groups of glucosamine, >99% of the free amino groups were blocked by acetylation and acetoacetylation, and there was 85% N-resulfation of totally desulfated heparin. The anticoagulant activity of the seven chemically modified heparins was measured by the activated partial thromboplastin time and thrombin time tests and was found, as reported by others (Casu, 1985), to be in all cases <1% of native heparin.

Binding of HBGF-1 to Different Chemically Modified Heparins. Initial experiments compared the ability of native heparin and its chemically modified variants to compete for the binding of HBGF-1 to heparin–agarose. HBGF-1 bound to heparin–agarose was quantified by SDS–PAGE and densitometer scanning of stained gels. Figure 1 demonstrates that

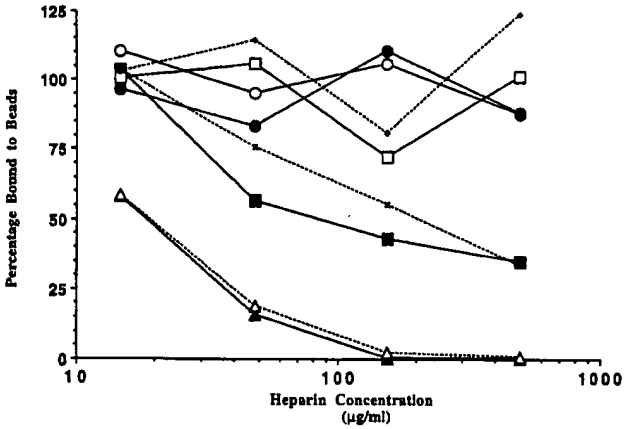

FIGURE 1: Inhibition of HBGF-1 binding to heparin–agarose by the indicated modified heparins as measured by SDS–PAGE and densitometer scanning of stained gels. Heparin (▲—▲) N-desulfated (○—○); N-desulfated, N-acetylated (×···×); N-desulfated, N-acetoacetylated (■—■); totally desulfated, N-acetoacetylated (●—●); totally desulfated (□—□); carboxyl-reduced (△···△); totally desulfated, N-resulfated (◆···◆). Values from each gel were normalized to the average of four HBGF-1 standards and are expressed as percentage of control binding. Each point represents the mean of duplicate determinations.

Table I: ED_{50} of Modified Heparins on Survival of Ciliary Neurons and Proliferation of 3T3 and HUVE Cells[a]

heparin prepn	ED_{50} of heparin (μg/mL)		
	HUVE	3T3	neurons
heparin	2	5	70
N-desulfated	>100	100	5
N-desulfated, N-acetylated	58	>100	12
N-desulfated, N-acetoacetylated	58	100	NA
totally desulfated, N-acetoacetylated	NA	NA	65
totally desulfated	NA	NA	25
carboxyl-reduced	60	37	9
totally desulfated, N-resulfated	NA	100	40

[a] Dose response curves were obtained for the modified heparins in the presence of a constant concentration (10 ng/mL) of beef heart HBGF-1. The dose required for 50% of the maximal stimulation observed was calculated by regression analysis of the curves. NA = not active.

the native and carboxyl-reduced heparins compete for binding of HBGF-1 with equal relative affinities; i.e., 50% inhibition was achieved at 15 and 17 μg/mL, respectively. N-Desulfated heparin did not inhibit binding of HBGF-1 at concentrations up to 500 μg/mL. Acetylation or acetoacetylation of the N-position of the glucosamine residues partially restored inhibitory capacity although these species were still approximately 10-fold less effective at competing with HBGF-1 binding to heparin–agarose than native heparin (50% inhibition at 170 μg/mL for N-acetylated and 140 μg/mL for N-acetoacetylated heparin). Totally desulfated; totally desulfated, N-acetoacetylated; and totally desulfated, N-resulfated heparins all failed to inhibit HBGF-1 binding over the concentration range tested.

Potentiation of Biological Activity of HBGF-1 by Chemically Modified Heparins. (A) Proliferation of HUVE Cells. The ability of the modified heparins to potentiate HBGF-1-induced proliferation of HUVE cells is shown in Figure 2. Native heparin is the most biologically active form of the molecule, potentiating the proliferative activity of HBGF-1 in log-linear fashion to 100 μg/mL with an ED_{50} of 2 μg/mL (Table I). Carboxyl-reduced heparin is less potent, 60 μg/mL being required to achieve the ED_{50} proliferation response of native heparin (Figure 2A). N-Desulfated heparin which had

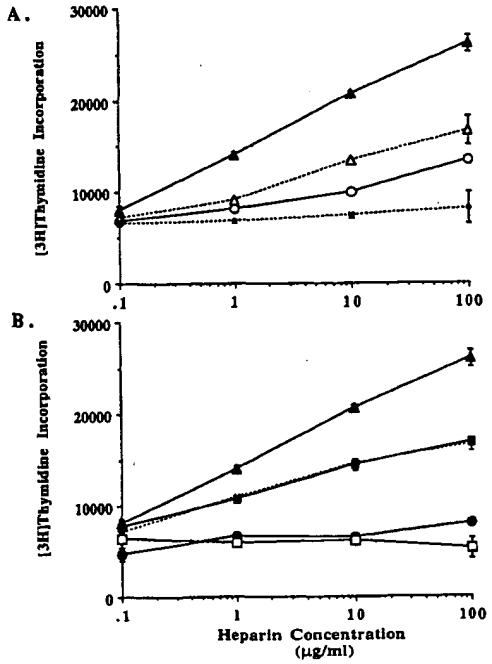

FIGURE 2: Effect of the modified heparins on HBGF-1-induced HUVE cell division. The dose response to the indicated modified heparin is shown in the presence of a constant concentration (10 ng/mL) of beef heart HBGF-1. [^{3}H]Thymidine incorporation is expressed as counts per minute. (A) Heparin (▲—▲); N-desulfated (○—○); carboxyl-reduced (△---△); totally desulfated, N-resulfated (♦---♦). (B) Heparin (▲—▲); N-desulfated, N-acetylated (×---×); N-desulfated, N-acetoacetylated (■—■); totally desulfated (□—□); totally desulfated, N-acetoacetylated (●—●). Values represent means and standard errors (n = 4) of one representative experiment. Identical results were obtained on three separate occasions.

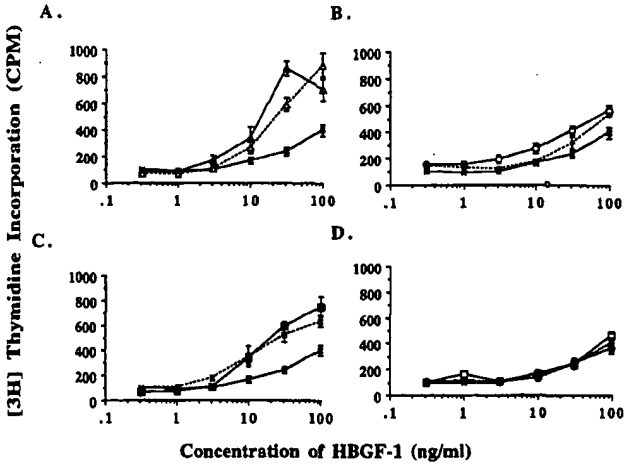

FIGURE 3: Effect of the modified heparins on HBGF-1-induced Balb/c3T3 cell division. A dose response to HBGF-1 is shown in the presence of a constant concentration (50 μg/mL) of the indicated modified heparin. [^{3}H]Thymidine incorporation is expressed as 10^{-2} cpm. (A) Control (×—×); heparin (▲—▲); carboxyl-reduced (△---△). (B) Control (×—×); N-desulfated (○—○); totally desulfated, N-resulfated (♦---♦). (C) Control (×—×); N-desulfated, N-acetylated (×---×); N-desulfated, N-acetoacetylated (■—■). (D) Control (×—×); totally desulfated (□—□); totally desulfated, N-acetoacetylated (●—●). Values represent means and standard errors of four experiments.

been either acetylated or acetoacetylated exhibited similar biological activity to carboxyl-reduced heparin with 58 μg/mL required to potentiate HBGF-1 to a degree equivalent to the ED_{50} for heparin (Figure 2B). The N-desulfated heparin was much less active although significantly potentiated HBGF-1 activity at high concentrations (Figure 2B). In contrast, the totally desulfated; totally desulfated, N-acetoacetylated; and totally desulfated, N-resulfated forms were inactive.

(B) Proliferation of Balb/c 3T3 Cells. The ability of the modified heparins to potentiate HBGF-1-induced proliferation of 3T3 cells is shown in Table I. Native heparin is the most biologically active form of the molecule with an ED_{50} of 5 μg/mL, the carboxyl-reduced form was the next most potent with an ED_{50} of 37 μg/mL, and the remaining modified heparins were much less active. The potentiating effects of the modified heparins were confirmed by determining the dose response of 3T3 cells to HBGF-1 in the presence of a constant concentration (50 μg/mL) of modified heparin. Given that none of the heparins was inhibitory at this high concentration, it provided an accurate determination of the potentiating activity of the modified heparins. Dose response curves for HBGF-1 in the presence of the given modified heparin are shown in Figure 3A–D. In agreement with previous studies, addition of heparin resulted in a significant shift of the dose response curve to the left. A lesser degree of potentiation was seen with the addition of the carboxyl-reduced molecule (Figure 3A). N-Desulfation of the heparin chain diminished its capacity to augment HBGF-1-induced mitosis although this preparation increased the activity of HBGF-1 at every concentration tested (Figure 3B). Acetylation or acetoacetylation of the desulfated N-position in the heparin chain increased its potentiating ability although not to the level of activity

Table II: Effect of Modified Heparins on Survival of Ciliary Neurons[a]

heparin prepn	cell no. at heparin concn (μg/mL) of 10	100
native heparin	396 ± 34	385 ± 12
N-desulfated	517 ± 28	671 ± 17
N-desulfated, N-acetylated	418 ± 42	539 ± 58
N-desulfated, N-acetoacetylated	396 ± 35	396 ± 44
totally desulfated, N-acetoacetylated	319 ± 16	605 ± 8
totally desulfated	407 ± 46	594 ± 20
carboxyl-reduced	440 ± 23	583 ± 31
totally desulfated, N-resulfated	385 ± 46	473 ± 13
control (no heparin)	264 ± 19	

[a] Effect of the modified heparins on HBGF-1-promoted ciliary neuronal survival. The survival response to the modified heparin is shown in the presence of a constant concentration (25 ng/mL) of beef heart-derived HBGF-1. Pooled data from four to six experiments are expressed as the total number ± standard error of survival neurons per well.

observed with the native molecule (Figure 3C); i.e., HBGF-1 activity in the presence of the N-acetylated or N-acetoacetylated molecules is similar to that in the presence of the carboxyl-reduced heparin. The totally desulfated, N-acetoacetylated and the totally desulfated heparins possessed no growth augmenting capacity (Figure 3D). N-Resulfation of the desulfated molecule produced a heparin species that slightly potentiated HBGF-1 at all concentrations tested (Figure 3B).

(C) Survival of Ciliary Neurons. The ability of the modified heparins to potentiate the neurotrophic activity of HBGF-1 was, in general, less than their ability to augment mitogenic activity. Tables I and II show that the neurotrophic activity of HBGF-1 for ciliary neurons is potentiated by all heparin species, although the native molecule is less active in enhancing HBGF-1 activity for ciliary neurons than some of the modified derivatives. Most active were the heparin molecules that were N-desulfated, carboxyl-reduced, or totally denuded of sulfate groups. These species exhibited a dose-related potentiation of HBGF-1 activity peaking around (2–2.5) × control at 100 μg/mL. There was no neuronal

survival in cultures containing the heparin preparations without HBGF-1.

Discussion

Using a range of chemically modified heparins, this study aimed to establish the structural features of heparin which play a critical role in HBGF-1 binding and potentiation of HBGF-1 action. In terms of HBGF-1 binding, the observation that carboxyl-reduced heparin bound HBGF-1 as effectively as the native heparin molecule indicated that the carboxyl groups of the uronic acid residues play little or no role in HBGF-1 binding. In contrast, totally desulfated heparin failed to interact with HBGF-1, implying that sulfate groups are essential for HBGF-1 binding. N-Desulfated heparin also lacked HBGF-1-binding capacity, and substitution of the exposed amino group with acetyl or acetoacetyl groups only partially restored binding capacity, indicating that *N*-sulfates play some role in growth factor binding. However, the failure of totally desulfated, N-resulfated heparin to interact with HBGF-1 demonstrated that *N*-sulfates alone are insufficient and ester sulfates are absolutely essential for HBGF-1 binding although whether the positioning of the ester sulfates on the polysaccharide backbone is of critical importance remains to be determined. The structural requirements for HBGF-1 binding to heparin are similar to those reported for the inhibition by heparin of complement C3 convertase (Kazatchkine et al., 1981) and the inhibition of smooth muscle cell proliferation (Castellot et al., 1985).

The ability of heparin to enhance HBGF-1-induced mitosis is well documented (Gospodarowicz & Cheng, 1986). Our results using 3T3 and HUVE cells are in line with those previously reported. However, the ability of the modified heparins to potentiate the mitogenic activity of HBGF-1 correlates only to a limited extent with their affinity for HBGF-1. The carboxyl-reduced molecule displayed similar affinity for HBGF-1 compared to the native heparin, although was consistently less potent in augmenting mitogenesis. The N-acetylated and the N-acetoacetylated species, although possessing less affinity for HBGF-1 than the carboxyl-reduced molecule, conferred similar biological activity to HBGF-1 for both 3T3 and HUVE cells. Moveover, N-desulfated heparin, although exhibiting no binding activity, increased the mitogenic activity of HBGF-1 for both 3T3 and HUVE cells above that seen with HBGF-1 alone. To a lesser extent, the totally desulfated, N-resulfated heparin potentiated HBGF-1 activity over 3T3 cells, an effect not seen with HUVE cells, whereas this heparin species failed to compete for HBGF-1 binding at concentrations up to 500 μg/mL. The totally desulfated, N-acetoacetylated and the totally desulfated heparins neither competed for HBGF-1 binding nor potentiated HBGF-1 above control levels.

Potentiation of neurotrophic activity revealed further variation in the activity of the modified heparin species. Indeed, heparin species without activity in the mitogenic assays were effective in promoting the neurotrophic activity of HBGF-1. In fact, removal of all sulfate groups resulted in heparin preparations capable of potentiating neurotrophic activity. Taken together, these results suggest additional mechanisms of HBGF-1 activation apart from those based entirely on a heparin–HBGF-1 association.

The observed variation in the biological activity of the modified heparins may be the result of several factors. First, the assays are different as the neuronal survival bioassay is of shorter time course than the cell proliferation assays and depends on cell survival rather than induction of DNA synthesis. Second, heparin may potentiate HBGF-1 action by protecting it from proteolysis, an effect which may be cell-dependent. There is ample evidence that heparin–HBGF-1 complexes are resistant to proteolysis (Gospodarowicz & Cheng, 1986; Rosengart et al., 1988) but the modified heparins that do not bind HBGF-1 could not potentiate growth factor action by this mechanism. Third, HSPG are known to be involved in cell attachment and spreading (Culp et al., 1980). Differential effects of the modified heparins on this aspect of cell behavior may account for some of the observed differences in activity. Neurons must first attach to the tissue culture substratum in order to survive, and it is possible that such mechanical factors are influenced to different degrees by the modified heparins. Early reports indicated that heparan sulfate and heparin were nonpermissive substrates for neuronal attachment and neurite outgrowth (Manthorpe et al., 1983) although more recently it was reported that an HSPG increased neurite elongation by dissociated E14 rat spinal neurons (Hantaz-Ambroise et al., 1987). Finally, it seems likely that heparin and its active derivatives act directly on target cells at a site distinct from the HBGF-1 receptor (Gospodarowicz & Cheng, 1986; Neufeld et al., 1987). In this context, cell-surface-binding sites for heparin have been described on smooth muscle cells (Castellot et al., 1985), hepatocytes (Kjellen et al., 1977), endothelial cells (Glimelius et al., 1978), fibroblasts (Chong & Parish, 1986), and neurons (Vidovic et al., 1986) as well as on cells of the reticuloendothelial system including macrophages and monocytes (Chong & Parish, 1986) and lymphocytes (Parish & Snowden, 1985). Furthermore, heparin has been shown to act on smooth muscle cells via cell-surface receptors to inhibit division both in vivo (Clowes & Karnovsky, 1977) and in vitro (Reilly et al., 1986). The recent demonstration that cell-surface-associated HSPG are essential for basic FGF action supports this view (Yayon et al., 1991; Rapraeger et al., 1991) although whether a similar situation exists with HBGF-1 remains to be clarified. In fact, recent studies have shown that both heparin and carboxyl-reduced heparin have no detectable effect on the high-affinity binding of radiolabeled HBGF-1 to fibroblasts (K. Brown and C. Parish, unpublished results).

Several other studies have shown that the simple association of heparin with growth factors does not account for all the reported potentiating effects of heparin on growth factor activity. For example, heparin, heparan sulfate, and chondroitin sulfate have been reported to potentiate the neurotrophic effects of NGF on PC12 cells (Neufeld et al., 1987; Damon et al., 1988) although heparin does not bind NGF (Watters and Hendry, unpublished observation). Heparin has also been reported to have differential effects on adrenal chromaffin cells, potentiating the neurite outgrowth response to HBGF-1, but having no effect on HBGF-1-induced mitogenesis (Claude et al., 1988). The present study has shown that the ability of modified heparin species to potentiate the biological activity of HBGF-1 is not only a direct function of their ability to bind HBGF-1. It is proposed that some of the biological activity of heparin is due to the direct action of heparin on cells possibly via heparin-specific receptors on the cell surface.

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References

Anderson, M. J., & Fambrough, D. M. (1983) *J. Cell Biol. 97*, 1396–1411.

Bonyhady, R. E., Hendry, I. A., Hill, C. E., & McLennan, I. S. (1980) *Neurosci. Lett. 18*, 197–201.

Bonyhady, R. E., Hendry, I. A., & Hill, C. E. (1982) *J. Neurosci. Res. 7*, 11–21.
Burgess, W. H., & Maciag, T. (1989) *Annu. Rev. Biochem. 58*, 575–606.
Carey, D. J., Rafferty, C. M., & Todd, M. S. (1987) *J. Cell Biol. 105*, 1013–1021.
Castellot, J. J., Jr., Wong, K., Herman, B., Hoover, R. L., Albertini, D. F., Wright, T. C., Caleb, B. L., & Karnovsky, M. J. (1985) *J. Cell. Physiol. 124*, 13–20.
Casu, B. (1985) *Adv. Carbohydr. Chem. Biochem. 43*, 51–134.
Chandrasekaran, E. V., & BeMiller, J. N. (1980) *Methods Carbohydr. Chem. 8*, 88–96.
Chong, A. S.-F., & Parish, C. R. (1986) *Immunology 58*, 277–284.
Claude, P., Parada, I. M., Gordon, K. A., D'Amore, P. A., & Wagner, J. A. (1988) *Neuron 1*, 783–790.
Clowes, A. W., & Karnovsky, M. J. (1977) *Nature 265*, 625–626.
Cole, G. J., Loewy, A., & Glaser, J. (1986) *Nature 320*, 445–447.
Culp, L. A., Ansbacher, R., & Domen, C. (1980) *Biochemistry 19*, 5899–5907.
Damon, D. H., D'Amore, P. A., & Wagner, J. A. (1988) *J. Cell. Physiol. 135*, 293–300.
Damon, D. H., Lobb, R. R., D'Amore, P. A., & Wagner, J. A. (1989) *J. Cell. Physiol. 138*, 221–226.
Farquhar, M. G. (1981) in *Cell Biology of the Extracellular Matrix* (Hay, E. D., Ed.) pp 335–378, Plenum, New York.
Fritze, L. M. S., Reilly, C. F., & Rosenberg, R. D. (1985) *J. Cell Biol. 100*, 1041–1049.
Glimelius, B., Busch, C., & Hook, M. (1978) *Thromb. Res. 12*, 773–782.
Gordon, P. B., Choi, H. U., Conn, G., Ahmed, A., Ehrmann, B., Rosenberg, L., & Hatcher, V. B. (1989) *J. Cell. Physiol. 140*, 584–592.
Gospodarowicz, D., & Cheng, J. (1986) *J. Cell. Physiol. 128*, 475–484.
Hantaz-Ambroise, D., Vigny, M., & Koenig, J. (1987) *J. Neurosci. 7*, 2293–2304.
Irimura, T., Nakajima, M., & Nicolson, G. L. (1986) *Biochemistry 25*, 5322–5328.
Kazatchkine, M. D., Fearon, D. T., Metcalfe, D. D., Rosenberg, R. D., & Austin, K. F. (1981) *J. Clin. Invest. 67*, 223–228.
Kjellen, L., Oldberg, A., Rubin, K., & Hook, M. (1977) *Biochem. Biophys. Res. Commun. 74*, 126–133.
Laurie, G. W., Bing, J. T., Kleinman, H. K., Hassell, J. R., Aumailley, M., Martin, G. R., & Feldman, R. J. (1986) *J. Mol. Biol. 189*, 205–216.
Lobb, R. R. (1988) *Biochemistry 27*, 2572–2578.
Lobb, R. R., Alderman, E. M., & Fett, J. W. (1985) *Biochemistry 24*, 4969–4973.
Maciag, T., Mehlman, T., Friesel, R., & Schreiber, A. B. (1984) *Science 225*, 932–935.
Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F. M., Davis, G. E., & Varon, S. (1983) *J. Cell Biol. 97*, 1882–1890.
Marcum, J. A., & Rosenberg, R. D. (1989) *Ann. N.Y. Acad. Sci. 556*, 81–94.
Nagasawa, K., & Inoue, Y. (1980a) *Methods Carbohydr. Chem. 8*, 287–289.
Nagasawa, K., & Inoue, Y. (1980b) *Methods Carbohydr. Chem. 8*, 291–294.
Neufeld, G., Gospodarowicz, D., Dodge, L., & Fujii, D. K. (1987) *J. Cell. Physiol. 131*, 131–140.
Parish, C. R., & Snowden, J. M. (1985) *Cell. Immunol. 91*, 201–214.
Parish, C. R., Coombe, D. R., Jakobsen, K. B., Bennett, F. A., & Underwood, P. A. (1987) *Int. J. Cancer 40*, 511–518.
Rapraeger, A. C., Krufka, A., & Olwin, B. B. (1991) *Science 252*, 1705–1708.
Reilly, C. F., Fritze, L. M. S., & Rosenberg, R. D. (1986) *J. Cell. Physiol. 129*, 11–19.
Rosengart, T. K., Johnson, W. V., Friesel, R., Clark, R., & Maciag, T. (1988) *Biochem. Biophys. Res. Commun. 152*, 432–440.
Schreiber, A. B., Kenney, J., Kowalski, W. J., Friesel, R., Mehlman, T., & Maciag, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6138–6142.
Sudhalter, J., Folkman, J., Svahn, C. M., Bergendal, K., & D'Amore, P. A. (1989) *J. Biol. Chem. 264*, 6892–6897.
Taylor, R. L., Shively, J. E., & Conrad, H. E. (1976) *Methods Carbohydr. Chem. 7*, 149–151.
Terranova, V. P., DiFlorio, R., Lyall, R. M., Hic, S., Friesel, R., & Maciag, T. (1985) *J. Cell Biol. 101*, 2330–2334.
Unsicker, K., Reichert-Preibsch, H., Schmidt, R., Pettmann, B., Labourdette, G., & Sensenbrenner, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5459–5463.
Vidovic, M., Hill, C. E., Hendry, I. A., & Parish, C. R. (1986) *J. Neurosci. Res. 15*, 503–511.
Watters, D. J., & Hendry, I. A. (1987) *J. Neurochem. 49*, 705–713.
Weigele, M., De Bernardo, S. L., & Tengi, J. P. (1972) *J. Am. Chem. Soc. 94*, 5927–5928.
Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991) *Cell 64*, 841–848.

Carbohydrate Research, 241 (1993) 249–259
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Note

Structure elucidation of a novel acidic tetrasaccharide and hexasaccharide derived from a chemically modified heparin *

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In the course of development of potentially more bioactive forms of heparin[1] for use as an anticoagulant, we had occasion to prepare a modified heparin first reported by Jaseja et al.[2]. Treatment of unfractionated bovine lung heparin with an alkaline solution at high temperature (105–110°C) for 24 h, according to method developed by Rej and Perlin[3], results in a modified heparin. To structurally characterize the sample of modified heparin, it was depolymerized with heparinase (heparin lyase I, EC 4.2.2.7). This resulted in the isolation of the hitherto unreported tetrasaccharide and hexasaccharide. This note describes the isolation and structure elucidation of two novel oligosaccharides. The solution conformation of these oligosaccharides may aid in developing a better understanding of the conformational preferences of the uronic acid residues.

Treatment of heparin with a sodium carbonate solution according to the method developed by Perlin and co-workers[2,3] results in a modified heparin. However, because heparin is a polydisperse microheterogeneous mixture, the modified heparin is also a mixture, and thus its precise structure was ill-defined. The ^{1}H NMR spectrum of the intact modified polymer indicated the presence of

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* Abbreviations include the following: SAX-HPLC, strong anion-exchange high performance liquid chromatography; FABMS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PAGE, polyacrylamide gel electrophoresis; TSP, sodium trimethylsilylpentanoate-2,2,3,3,4,4-d_6; AUFS, absorbance units full scale, NOE, nuclear Overhauser effect; 2D, two dimensional; COSY, correlated spectroscopy; HETCOR, heteronuclear correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; FID, free-induction decay.

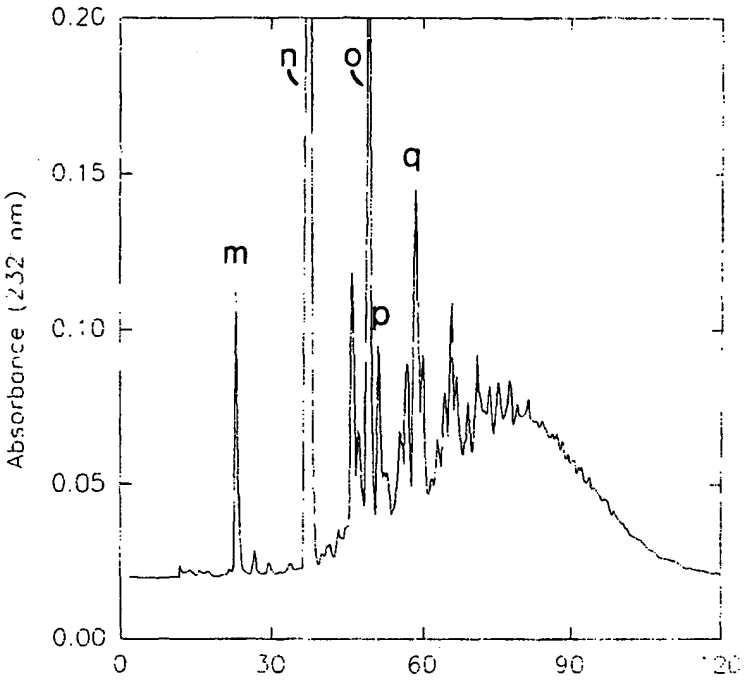

Fig. 1. SAX-HPLC chromatogram of the heparin lyase I depolymerized, modified, heparin sample.

modified uronic acid residues, but it also showed the presence of at least 25% of intact α-L-idopyranosyluronic acid residues. To better define the structure of the modified polymer, it was treated with heparin lyase I, and enzymic depolymerization was monitored by UV spectroscopy. When the reaction had reached ~21% completion no further depolymerization was observed. This result is consistent with the reduced sensitivity of this modified heparin towards heparinase reported by Rej and Perlin[2]. Gradient PAGE analysis of the heparinase-depolymerized, modified heparin indicated the presence of significant quantities of higher oligosaccharides in addition to the expected di-, tetra-, and hexa-saccharides. Five oligosaccharides (peaks m, n, o, p, and q in Fig. 1) could be isolated by SAX-HPLC representing 1.2% of the total mass of the modified polymer. Analysis of the oligosaccharides, corresponding to peaks m, n, o, p, and q, using analytical SAX-HPLC showed each to have a purity > 95%.

Peaks m, n, and p were subjected to analyses by ^{1}H NMR spectroscopy, and the spectra for these compounds were found to be superimposable with the spectra of compounds **1**, **2**, and **3** (Fig. 2) already reported by our group[4]. Compounds **1**, **2**, and **3** represent residues in the heparin chain which have escaped modification and account for ~50% of the isolable mass. Peaks o and q had distinctly different retention times on the SAX-HPLC column as compared with the retention times of known standards prepared in our laboratory[5,6]. Their elution profiles suggested

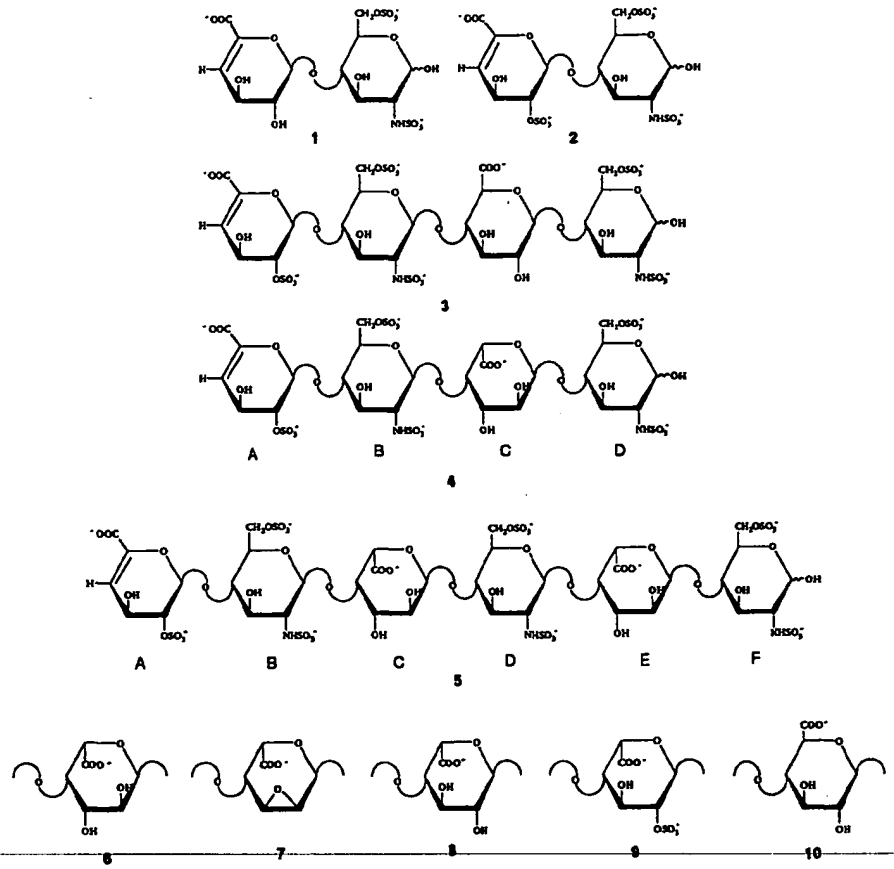

Fig. 2. Oligosaccharides (1–5) derived by the action of heparin lyase I (EC 4.2.2.7) on modified heparin substrate. Structures 6–10 have been reported in modified heparin[2,3].

that they were a tetrasaccharide and hexasaccharide with five and seven sulfate groups, respectively. The ^{1}H NMR spectrum of the peak o (Fig. 3a) clearly indicated the presence of four anomeric protons in addition to the proton signal at C-4 of the unsaturated uronic acid residue at the nonreducing terminus. Absence of the acetate CH_3 signal indicated that the compound was an *N*-sulfated tetrasaccharide. A homonuclear COSY spectrum provided the necessary connectivities for the assignment of all protons. Starting from the most deshielded proton of the unsaturated uronic acid at the nonreducing end (Fig. 4), the complete spin system for the A-ring could be identified. Similarly, the spin systems associated with the glucosamine and the internal uronic acid residues could also be identified. Sequence information was obtained through a transient NOE experiment (Fig. 5) in which the interresidue protons at the 1- and 4-positions could be identified. H-1 of the C-ring at 5.22 ppm showed a through-space coupling with a proton at 3.70 ppm corresponding to H-4 of the D-ring. Similarly, H-1 of the B-ring was found to

have a dipolar coupling with proton H-4 of the C-ring. Although this NOE experiment resulted in sequence information, it was not possible to determine the precise structure of the internal uronic acid residue. In their studies on modified heparin, Rej and Perlin[3] report the formation of either an epoxy uronic acid derivative (structure 7, Fig. 2), or inversion of stereochemistry at the 2- and 3-positions (structure 6), or a uronic acid residue having retention of the stereochemistry with the loss of a 2-sulfate group (structure 8). The chemical shift of the H-1 of the C-ring (5.22 ppm) clearly showed that neither a nonsulfated idopyranosyluronic (8) nor a glucopyranosyluronic (10) acid residue is present in 4. Since 2-sulfation causes the deshielding of the anomeric proton in uronic acids by ~ 0.2 ppm, a 2-sulfated α-L-idopyranosyluronic acid residue (structure 9) could be ruled out. An inverse heteronuclear $^{1}H-^{13}C$ correlation (HETCOR) experiment (Fig. 6) showed that C-2 and C-3 of the internal uronic acid residue resonated at 72.5 and 73.7 ppm, respectively (Table I). The low chemical shift dispersion in the ^{1}H and ^{13}C signals at the 2-position and the 3-position of the internal uronic acid, together with the chemical shift of H-1 (5.22 ppm), suggested that the internal uronic acid was α-L-galactopyranosyluronic acid (6) and the structure of peak o (Fig. 1) was probably 4. The presence of this residue was confirmed by a NOESY experiment (Fig. 5), which shows a weak crosspeak associated with protons at the 5-position and the 3-position, in addition to the expected dipolar couplings between H-4 and H-5, H-1 and H-2, and H-4 and H-3 of the C-ring. The absence of a dipolar coupling between H-2 and H-3 of the C-ring also suggests an *antiperiplanar* arrangement for the two protons. The ^{3}J coupling constant for H-1 of the C-ring is approximately 2.2 Hz implying a *synclinal* orientation of protons at the 1- and 2-positions. Since the stereochemical configurations of C-1 and C-2 of the C-ring are expected to remain unchanged in the chemical transformation, and the NOESY experiment establishes the relative stereochemistry of protons at the 2-, 3-, 4-, and 5-positions, the configuration of the internal uronic acid residue was established as α-L-galactopyranosyluronic acid. The negative ion FABMS spectrum of the compound showed the $[M-Na]^{+}$ ion peak at m/z 1205 ruling out the possibility of the epoxy derivative (structure 7) which would have shown peaks at either m/z 1209 $[M]^{+}$ or 1187 $[M-Na]^{+}$. The ^{1}H NMR spectrum (Fig. 3b) of peak q in the SAX-HPLC, showed the presence of six signals that could be ascribed to anomeric protons, indicating it to be a hexasaccharide. Concerted application of 2D NMR techniques including COSY, inverse heteronuclear correlation, NOESY, and ROESY, in a manner described for tetrasaccharide 4, led to the structured 5.

The conformational analysis of α-L-idopyranosyluronic acid (8 and 9) has been the subject of study[7,8] in order to elucidate special features in the pentasaccharide binding sequence. The conformation of uronic acid residues is known to be significantly affected by the presence of sulfate groups within the residue as well as on adjacent residues. α-L-Idopyranosyluronic acid (8, 9) exhibits conformational flexibility while β-D-glucopyranosyluronic acid (10) appears to be rather rigid[7,8].

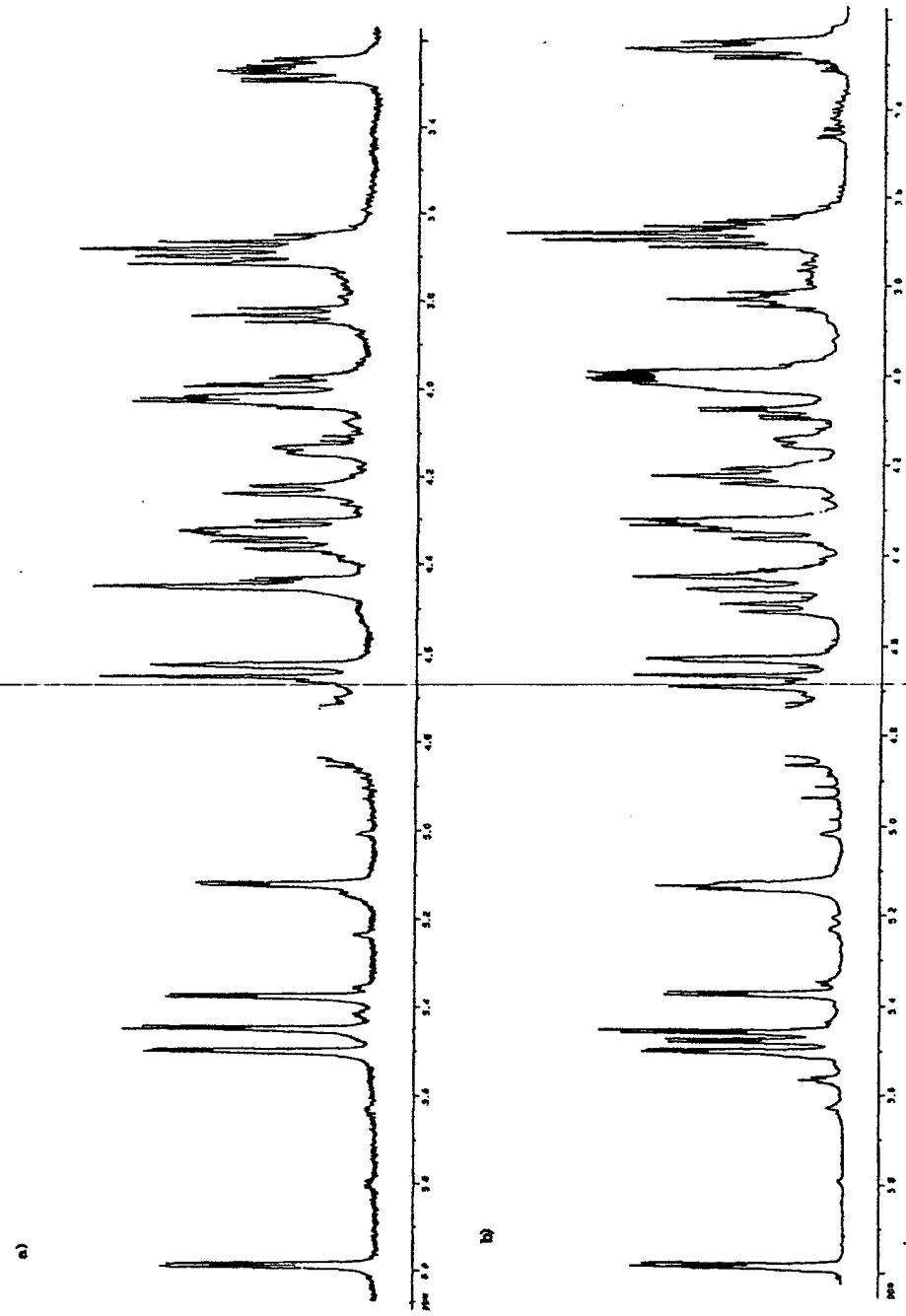

Fig. 3. ^{1}H NMR spectrum (600 MHz) of 4 (a) and 6 (b).

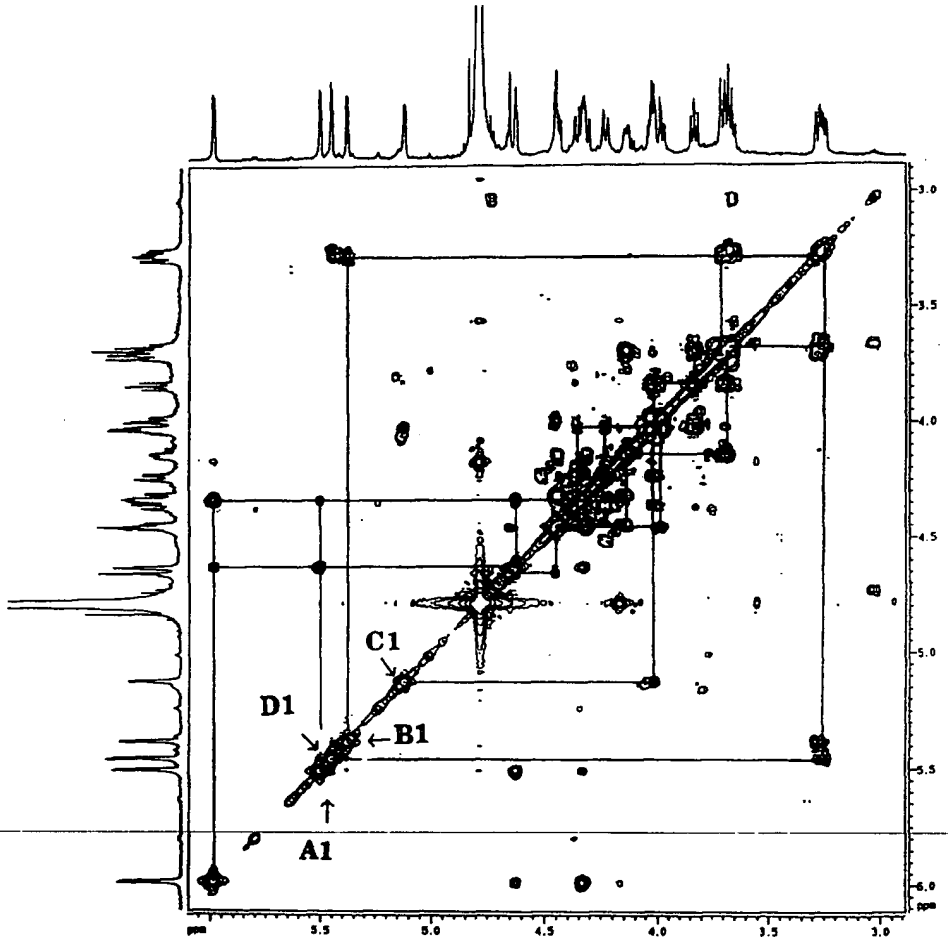

Fig. 4. The homonuclear COSY spectrum (600 MHz) for 4. The appropriate connectivities can be followed by the lines drawn from the appropriate H-1 signal of the different rings. (A1 = H-1 of the A-ring, etc.). The connectivity pattern for the H-2 and H-3 of the C-ring is clear even though the signal dispersion is very low. The digital resolution was 1.6 Hz/pt.

Hence, it was important to analyze the conformational preference of the α-L-galactopyranosyluronic acid residue (**6**) and compare the differences. On performing a 2D *J*-resolved NMR experiment, coupling constants for most of the protons in compound **4** were obtained (Table II). The 2D *J*-resolved spectrum for compound **5** could not be analyzed because of severe overlap due to nearly equivalent chemical shifts. The coupling constants for the H-2 and H-3 (~ 10 Hz) of the α-L-galactopyranosyluronic acid residue of tetrasaccharide **4** indicate that the protons are *antiperiplanar*. The ^3J coupling constants of H-1 (2.2 Hz) and H-4 (2.5 Hz) suggest that the substituents at these positions are axially oriented. Hence, the internal α-L-galactopyranosyluronic acid ring is conformationally biased towards

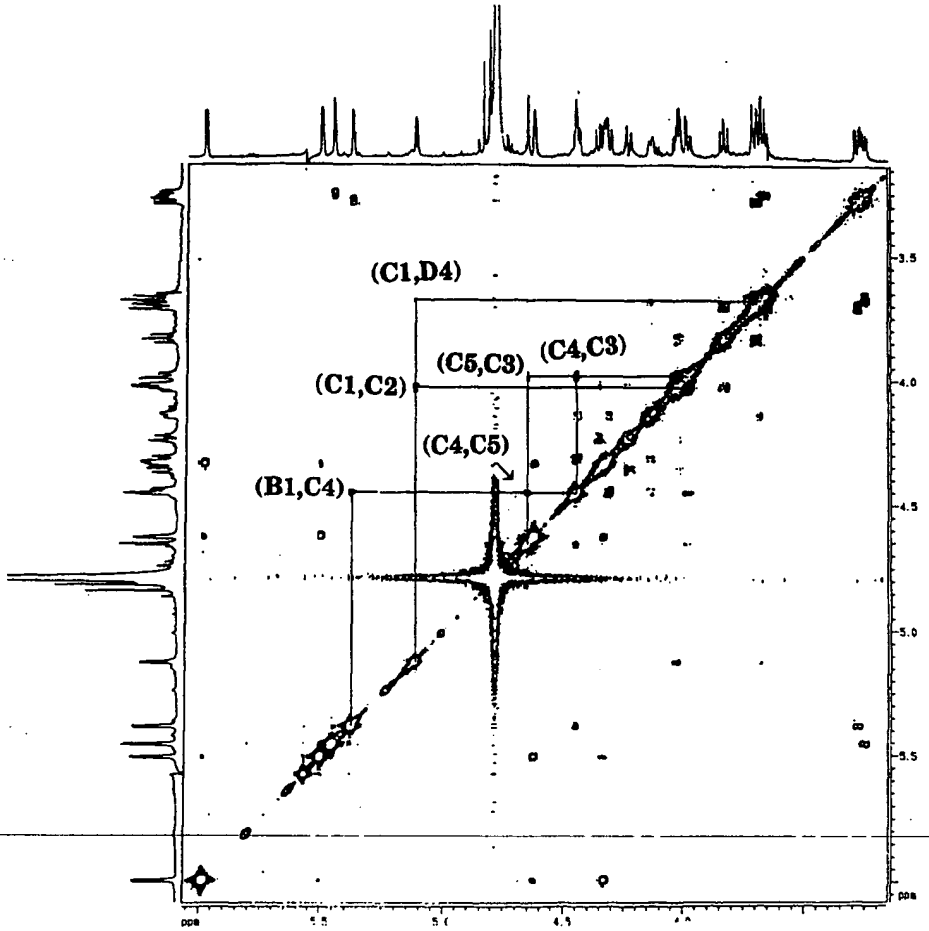

Fig. 5. The 2D transient NOE experiment for 4. A presaturation of the HO^2H signal was avoided so as to not obscure the connectivities of the C-ring protons. (Note B1 = H-1 of the B-ring, etc.). Note the clear difference in intensity between the (C5, C3) and the (C4, C3) crosspeaks. Also the crosspeak for C1, C2 clearly shows the *synclinal* nature of the two protons.

the $^1C_4$ chair form (Fig. 7). The discrepancy observed in the ^3J coupling constants of H-1 and H-2 of the C-ring (Table II), probably arises due to the second ordered nature of these couplings, or due to the presence of a distorted $^1C_4$ chair form of an α-L-galactopyranosyluronic acid ring in relatively reduced proportion. The presence of the $^4C_1$ chair conformation, however, can be ruled out, by the ^3J coupling constant analysis. This conformational preference of the unsulfated α-L-galactopyranosyluronic acid residue for the $^1C_4$ chair form suggests that the presence of a sulfate group at the 2-position on the α-L-idopyranosyluronic acid residue brings about the conformational change to a distorted $^1C_4$ chair form rather than the expected $^4C_1$ chair form.

EXPERIMENTAL

Materials and methods.—Heparin (sodium salt) from bovine lung (167 U/mg), was obtained from Sigma Chemical Co., St. Louis, MO. Heparin lyase I (EC 4.2.2.7) was purified from *Flavobacterium heparinum* (5 mIU/mg)[9] or purchased (11 mIU/mg) from Sigma Chemical Co., St. Louis, MO. Sulfopropyl (SP) Sephadex C-50 was purchased from Sigma Chemical Co., St. Louis, MO, while Bio-Gel P-2 was from Bio-Rad Laboratories, Richmond, CA. SAX-HPLC was performed using a Shimadzu LC-7A dual pump system, and the detection system consisted of an

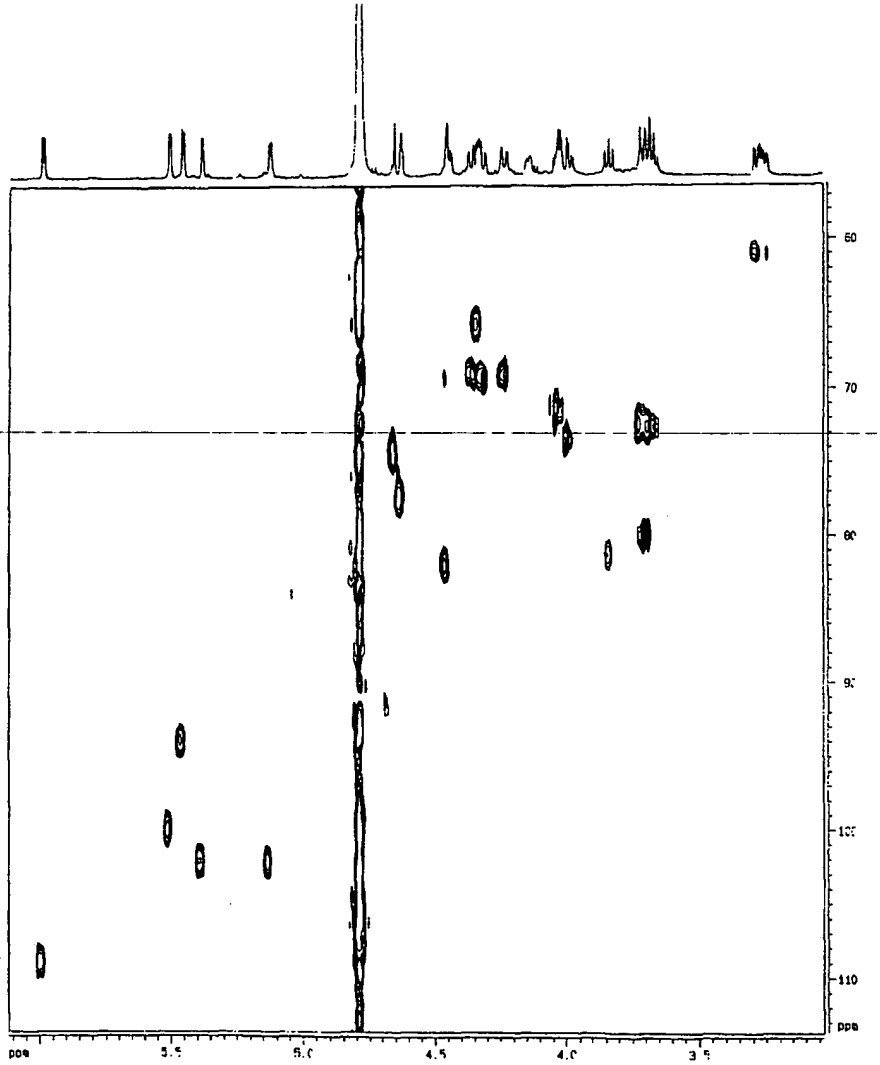

Fig. 6. The 2D inverse heterocorrelation (HETCOR) experiment for 4.

TABLE I [a]

The ^{1}H and ^{13}C NMR chemical shifts of **4** and **5**

4					5						
Ring proton	A	B	C	D	Ring proton	A	B	C	D	E	F
1	5.51	5.37	5.22	5.45	1	5.50	5.48	5.14	5.38	5.12	5.46
2	4.62	3.27	4.05	3.26	2	4.63	3.25	4.09	3.30	3.98	3.27
3	4.34	3.75	4.00	3.67	3	4.33	3.67	3.99	3.64	4.00	3.70
4	5.99	3.83	4.46	3.70	4	5.99	3.69	4.47	3.70	4.44	3.69
5		4.02	4.62	4.13	5		4.14	4.67	4.02	4.69	4.02
6		4.23		4.32	6		4.32		4.23		4.21
6′		4.35		4.44	6′		4.43		4.35		4.51
Carbon					Carbon						
1	100.2	102.2	102.1	94.2	1	101.59	102.74	103.53	103.64	103.53	95.50
2	77.5	61.1	72.5	60.9	2	78.94	62.93	no	62.93	nf	62.34
3	65.8	72.5	73.7	72.9	3	67.32	73.98	nf	73.98	nf	73.98
4	109	81.5	82	79.9	4	110.27	81.08	82.05	no	83.84	no
5	nf	71.5	74.2	71.5	5	no	nf	75.83	nf	75.90	nf
6	nf	69	nf	69.5	6	no	70.94	nf	70.54	nf	70.81

[a] No, not observed; nf, not found due to signal overlap

TABLE II

The $^{3}J_{H,H}$ vicinal coupling constant (Hz) for **4** derived from the *J*-resolved NMR experiment

Ring proton	A	B	C	D
1	1.38, 3.08	3.52	2.19	3.6
2	1.38, 5.54	10.46, 3.69	4.00, 10.31	3.53, 8.31
3	4.66, 5.24, 3.08	8.31, 10.61	2.77, 10.05	9.54
4	4.66, 1.21	8.62, 10.16	2.46	10.01
5		10.31	2.37	m [a]
6		3.94, 10.62		10.62, 1.38
6′		1.69, 10.54		10.46, 1.38

[a] Multiplet not analyzable

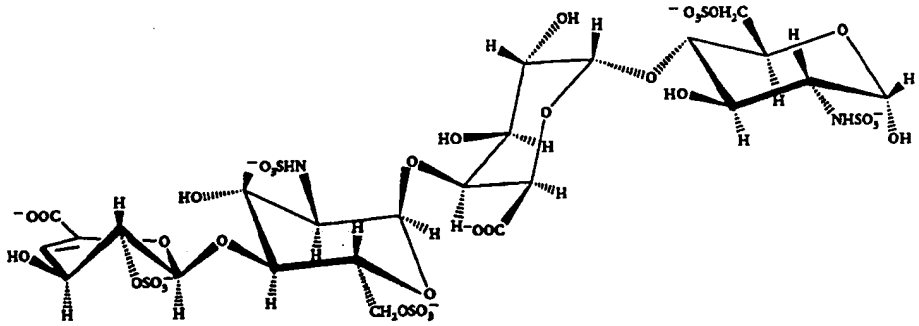

Fig. 7. The $^{1}C_{4}$ conformation of α-L-galactopyranosyluronic acid residue and a probable global conformation for **4** based on literature reports of the preferred conformations[7,8].

LKB Bromma 2141 variable wavelength detector operating at 232 nm. The injection system was a Rheodyne 7125 injector with a Spherisorb SAX-HPLC column from Phase Separations, Norwalk, CT. UV spectroscopy was performed with a Shimadzu UV-160 spectrophotometer, and mass spectrometry was carried out with a VG Analytical ZAB-HF spectrometer using ethanolamine as the matrix.

All ^{1}H and ^{13}C NMR experiments on tetrasaccharide **4** and hexasaccharide **5** were performed on a Bruker AMX 600 MHz spectrometer equipped with an X32 computer. Each oligosaccharide (~2 mg) was dissolved in $^{2}H_2O$ (0.5 mL) exchanged thrice from 99.96% $^{2}H_2O$, and 0.03% (w/v) TSP was added as internal standard. The final solution was filtered through a 0.45-μm membrane. The 2D NMR experiments were performed using standard Bruker software at 298 K. The COSY experiment utilized $\pi/2$–t_1–$\pi/2$–FID pulse program. The number of data points in the F_1-dimension were 256, each obtained with 16 scans. The FIDs were Fourier-transformed onto a data matrix of 1K × 1K with a sine-bell window function. The NOESY experiment utilized a $\pi/2$–t_1–$\pi/2$–τ_m–$\pi/2$–FID pulse program with 512 t_1, increments acquired with 32 scans each. The FIDs were Fourier-transformed onto a data matrix of 1K × 1K with a phase shifted sine-bell window function and the two frequency domains symmetrized. A τ_m value of 800 ms was found to provide the required connectivities in the NOESY experiments. The mixing time was randomly varied by 5% to suppress the *J*-connectivities. The inverse heteronuclear correlation (HETCOR) experiment was performed using standard Bruker software with 128 t_1 increments, each acquired with 104 scans. A recovery delay of 4.25 s was employed. The FIDs were Fourier-transformed onto a data matrix of 1K × 512W using sine-bell window function.

Base-catalysed modification of heparin.—A solution of bovine lung heparin (1 g) and sodium carbonate (0.45 g) in water (50 mL) was heated for 24 h at 105–110°C in an oil bath. After cooling, the solution was exhaustively dialyzed for 72 h against deionized, distilled water and freeze-dried to yield 653 mg of the modified heparin.

Enzymatic depolymerization of modified heparin.—The modified heparin (500 mg in 25 mL) was exhaustively depolymerized for 70 h at 30°C with 2.5 IU (1 IU = 1 μmol product formed/min) heparin lyase I (EC 4.2.2.7) in a 5 mM sodium phosphate buffer containing 0.2 M NaCl at pH 7.0. The reaction was monitored by removing aliquots and measuring absorbance at 232 nm, after a 1:100 dilution in 30 mM HCl. A constant absorbance value in the presence of active enzyme indicated that the reaction was complete. The reaction was then terminated by heating for 1 min at 100°C. The mixture was adjusted to pH 2.5 with concd HCl and passed through a 15 × 0.5 cm SP Sephadex column to remove protein. After readjusting to pH 7.0, the same was desalted on a 35 × 2.5 cm Bio-Gel P-2 column, freeze-dried, and reconstituted at 100 mg/mL in deionized, distilled water for SAX-HPLC purification step.

Purification of the oligosaccharide mixture with SAX-HPLC.—A semipreparative SAX-HPLC column[4] was preequilibrated with 0.2 M NaCl at pH 3.5. The desalted, reconstituted, enzymically depolymerized sample was loaded (75–100 mg

each injection) and eluted using a linear gradient (concentration (y, in M) at any time (x in s) = 0.0015x + 0.2) of NaCl at pH 3.5 at a flow rate of 6–8 mL/min. The elution profile was monitored by absorbance at 232 nm (0.5–1.0 AUFS). Fractions having the same retention times from different runs were combined, freeze-dried, desalted on Bio-Gel P-2 column, and freeze-dried to obtain five major oligosaccharide products.

CONCLUSIONS

Treatment of heparin with sodium carbonate solution at high temperature leads to a modified polymer that could be depolymerized by heparin lyase I (EC 4.2.2.7) leading to a mixture of oligosaccharides. A novel tetrasaccharide and hexasaccharide with internal α-L-galactopyranosyluronic acid residues were isolated and characterized by 2D NMR techniques. Complete assignment of ^{1}H NMR spectra and partial assignment of ^{13}C NMR spectra of these oligosaccharides was carried out. The conformational preference of the α-L-galactopyranosyluronic acid residue was deduced to be $^1C_4$ as determined by NOE as well as coupling constants analyses.

ACKNOWLEDGMENTS

This research was supported by the N.I.H. grant no GM 38060 and by a predoctoral fellowship to H.M.W. from the University of Iowa Biocatalysis Center.

REFERENCES

1 R.J. Linhardt, *Chem. Ind. (London)*, 2 (1991) 45–50.
2 M. Jaseja, R.N. Rej, F. Sauriol, and A.S. Perlin, *Can. J. Chem.*, 67 (1989) 1449–1456.
3 R.N. Rej and A.S. Perlin, *Carbohydr. Res.*, 200 (1990) 437–447.
4 K.G. Rice and R.J. Linhardt, *Carbohydr. Res.*, 190 (1989) 219–233.
5 R.J. Linhardt, K.G. Rice, Z.M. Merchant, Y.S. Kim, and D.L. Lohse, *J. Biol. Chem.*, 262 (1986) 14448–14454.
6 R.J. Linhardt, H.M. Wang, D. Loganathan, and J.-H. Bae, *J. Biol. Chem.*, 267 (1992) 2380–2387.
7 G. Torri, B. Casu, G. Gatti, M. Petitou, J. Choay, and J.C. Jacquinet, *Biochem. Biophys. Res. Commun.*, 128 (1985) 134–140.
8 C.A.A. van Boeckel, S.F. van Aelst, G.N. Wagenaars, J.-R. Mellema, H. Paulsen, T. Peters, A. Pollex, and Y. Sinnwell, *Ec. Trav. Chim. Pays-Bas*, 106 (1987) 19–29.
9 D.L. Lohse and R.J. Linhardt, *J. Biol. Chem.*, 267 (1992) 24347–24355.

Carbohydr Res. 1994 Oct 17;263(2):271-84. Related Articles, Links

Heparin-like compounds prepared by chemical modification of capsular polysaccharide from E. coli K5.

Casu B, Grazioli G, Razi N, Guerrini M, Naggi A, Torri G, Oreste P, Tursi F, Zoppetti G, Lindahl U.

Istituto di Chimica e Biochimica G. Ronzoni, Milan, Italy.

O-Sulfation of sulfaminoheparosan SAH, a glycosaminoglucuronan with the structure-->4)-beta-D-GlcA(1-->4)-beta-D-GlcNSO3(-)-(1-->, obtained by N-deacetylation and N-sulfation of the capsular polysaccharide from E. coli K5, was investigated in order to characterize the sulfation pattern eliciting heparin-like activities. SAH was reacted (as the tributylammonium salt in N,N-dimethylformamide) with pyridine-sulfur trioxide under systematically different experimental conditions. The structure of O-sulfated products (SAHS), as determined by mono- and two-dimensional 1H and 13C NMR, varied with variation of reaction parameters. Sulfation of SAH preferentially occurred at O-6 of the GlcNSO3- residues. Further sulfation occurred either at O-3 or at O-2 of the GlcA residues, depending on the experimental conditions. Products with significantly high affinity for antithrombin and antifactor Xa activity were obtained under well-defined conditions. These products contained the trisulfated aminosugar GlcNSO3-3,6SO3-, which is a marker component of the pentasaccharide sequence through which heparin binds to antithrombin.

PMID: 7805054 [PubMed - indexed for MEDLINE]

1: Thromb Res. 1990 Jul 15;59(2):237-46. Related Articles, Links

Conventional heparin and semisynthetic heparin analogue (SSHA) alteration of blood coagulation after embolic occlusion of human renal circulation.

Torngren S, Blomback M, Almgard LE, Norming U, Nyman CR.

Department of Surgery, Sodersjukhuset, Stockholm, Sweden.

Blood coagulation and fibrinolytic variables were measured in a peripheral vein in a study of 21 consecutive patients before and after angio-embolization of renal carcinoma. The first ten patients received conventional heparin, 5000 IU twice daily, and the following eleven a semi-synthetic heparin analogue (SSHA), 50 mg twice daily, for 5 days. The first injection was given 2 hours before embolization and the last injection at least 12 hours before the last blood sampling. Both groups showed increased levels of FPA on day 5-7, indicating that the anticoagulant influence had ceased. F VII levels decreased only in the SSHA group from embolization to day 3, but were increased in both groups on day 5-7. Levels of thrombin-antithrombin complexes (TAT) were significantly increased in the heparin group 2 hours after embolization, indicating that thrombin activity had been formed. The corresponding TAT level in the SSHA group was not significantly increased. The differences could possibly indicate a different mechanism of action on blood coagulation of SSHA as compared with heparin, with involvement of extrinsic pathway and maybe by-passing antithrombin III inhibition.

1: Diabetes Res Clin Pract. 1993 Jul;21(1):5-9. Related Articles, L

The effect of a heparin analogue, ITF-5005, on diabetes incidenc and insulitis in the non-obese diabetic mouse.

Beales PE, Williams AJ, Signore A, Procaccini E, Xu S, Pozzilli P.

Department of Diabetes and Metabolism, St. Bartholomew's Hospital, West Smithfield, London, UK.

It has been suggested that heparin and its analogues may have a suppressive effect on the immune response by interfering with T-lymphocyte heparinase activity, thus altering the ability of T-lymphocytes to penetrate the extracellu matrix and migrate to target tissues. We have investigated whether a heparin analogue (ITF-5005) can alter lymphocytic infiltration of the endocrine pancreas (insulitis) and/or diabetes incidence in the non-obese diabetic (NOI mouse. Sixty-four NOD mice were divided at weaning and injected subcutaneously five times per week with either 18, 36 or 72 micrograms/kg body weight of ITF-5005 or saline as a control. At 12 weeks of age, the anim were culled and their pancreata sectioned, stained and assessed 'blind' for insulitis and insulin containing cells. Insulitis was similar in all groups as wa the proportion of insulin-containing cells. To determine the effect on diabete incidence, two groups of mice were injected with either saline or 140 micrograms/kg body weight of ITF-5005 from weaning until 30 weeks of ag No difference was found in overall diabetes incidence; however, disease ons was significantly accelerated in the treated group. We conclude that ITF-500 at the doses employed, has no effect on insulitis or the proportion of treated group. We conclude that ITF-5005, at the doses employed, has no effect on insulitis or the proportion of insulin-containing cells found in the pancreas, b that it can accelerate the course of diabetes in the NOD mouse.

PMID: 8253022 [PubMed - indexed for MEDLINE]

Bioorg Med Chem Lett. 1999 Apr 19;9(8):1155-60. Related Articles, Links

ELSEVIER SCIENCE
FULL-TEXT ARTICLE

New synthetic heparin mimetics able to inhibit thrombin and factor Xa.

Petitou M, Duchaussoy P, Driguez PA, Herault JP, Lormeau JC, Herbert JM.

Sanofi Recherche, Haemobiology Research Department, Toulouse, France.

Synthetic pentadeca-, heptadeca- and nonadecasaccharides, comprising an antithrombin III (AT III) binding pentasaccharide prolonged at the non-reducing end by a thrombin binding domain have been obtained. The pentadecasaccharide is the shortest oligosaccharide able to catalyse thrombin inhibition by AT III. The nonadecasaccharide is a more potent thrombin inhibitor than standard heparin.

Bioorg Med Chem Lett. 1998 May 19;8(10):1201-6. Related Articles, Links

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In vitro evaluation of synthetic heparin-like conjugates comprising different thrombin binding domains.

Basten JE, Dreef-Tromp CM, de Wijs B, van Boeckel CA.

N.V. Organon Scientific Development Group, The Netherlands.

The syntheses of several heparin-like glycoconjugates (i.e., 16a-f) containing identical AT III binding domains (ABD) and spacers but different thrombin binding domains (TBDs) are described. Biological activities of conjugates 16a-f indicate that the thrombin inhibitory activity is mainly determined by the charge density of the TBD moiety.

J Chromatogr A. 1998 Dec 18;828(1-2):497-508. Related Articles, Links

Use of reversed polarity and a pressure gradient in the analysis of disaccharide composition of heparin by capillary electrophoresis.

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A capillary electrophoresis method with reversed polarity, combining both the application of a voltage and a pressure gradient between the buffer vials, was developed for the analysis of eight heparin-derived delta-disaccharides obtained by enzymatic depolymerization. A 60 mM formic acid buffer at pH 3.40 was selected as running electrolyte, with an applied voltage of -15 kV and an over-imposed pressure gradient (3.45.10(-3) MPa) for 6 min from inlet to outlet starting at 20 min. Figures of merit such as run-to-run and day-to-day precision, and limits of detection were established. The electrophoretic method was applied to the analysis of depolymerization products of different kinds of heparins. The composition of the depolymerization buffer was selected in order to reduce baseline distortions in the electrophoretic separation, thus a buffer solution containing 20 mM Tris, 50 mM sodium chloride, and 3 mM calcium chloride at pH 7.10 was used. Percentages of molar disaccharide compositions for unfractionated heparins from porcine, bovine and ovine intestinal mucosa, and bovine lung were determined. In addition, low-molecular-mass heparins from bovine and porcine intestinal mucosa were analysed as well.

Pharmacotherapy. 1999 Sep;19(9 Pt 2):155S-160S. Related Articles, Links

Low-molecular-weight heparins for acute coronary syndromes: an emergency medicine perspective.

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Patients with chest pain represent one of the largest and most challenging populations for emergency departments to treat. Diagnostic and treatment modalities implemented in the emergency department are associated with significant clinical outcomes and financial implications. Critical pathways are being developed to increase the speed and efficiency with which these patients are managed. Of particular importance is the evolving role of low-molecular-weight heparins, which have both clinical and economic advantages over unfractionated heparins in treating unstable angina and non-Q wave myocardial infarction.